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Potential field validation sites were evaluated in June 1989. Soldier Creek, emanating from Tinker AFB, was selected because of its industrial/sewage effluent. Soldier Creek is the largest tributary of Crutcho Creek, which empties into the North Canadian River. Twelve sites along the three water ways were evaluated. Water was collected from each site and transported on ice. Toxicity was determined by placing 90d fathead minnows into 1-gallon jars filled with 3L of sample water. Two sites immediately below Tinker AFB exhibited acute toxicity with total mortality occuring within 30 minutes of test initiation. However, all other sites, showed no toxicity. The free chlorine level was in the site i-mediately below Tinker AFB (site 1) was 1.6 mg Cl_2/L, site 2 was 0.8 mg Cl_2/L, and 0.2 mg Cl_2/L at all other sites. Thus, a toxicity and chemical gradient was found in Soldier Creek. Sediment collected at four sites, closest to Tinker AFB, smelled of fuel and solvents. Sediment toxicity was evaluated by placing 90d fathead minnows into 1 gallon glass jars filled 600 ml of Soldier Creek sediment overlaid with 2400 ml of Soldier Creek water. No toxicity was seen in any of the stations. (SDW)

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ANNUAL TECHNICAL REPORT

EVALUATION OF THE EFFICACY OF THE STRESS PROTEIN RESPONSE AS A BIOCHEMICAL WATER QUALITY BIOMONITORING METHOD
AFOSR-88-0295

by

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University of North Texas
August 31, 1989

Project Objectives

The objective of this research is to develop a method for measuring cellular responses to stress that can be used to asses both levels and possible types of stressors in fish, with the potential for adapting the procedure as a rapid, precise water quality biomonitoring method. This study will evaluate the efficacy of using the phenotypic expression of the stress proteins, a cellular response that increases the organism's capacity to cope with greater stress loads, as a means of determining the degree of stress caused by anthropogenic contaminants in the aquatic environment.

During the first year of the project, two exposures have been completed and statistically analyzed: heat shock and sodium arsenite. Both experiments are currently being prepared for publication submittal. The following report describes the methods and results from the heat shock and arsenite experiments. In addition, preliminary findings from sodium chromate exposures and first phase field validation studies are presented.

Experimental Procedures

Ninety- to 120-day-old fathead minnows (Pimephales promelas) were obtained from a culture located at the University of North Texas. Fish used for the study were acclimated to 25°C for at least 7 days and a 16L:8D photoperiod and were fed frozen brine shrimp once daily to satiation.

Heat Shock Exposures

Three different heat shock experiments were performed: 1) an *in vitro* heat shock temperature-response experiment with striated muscle; 2) an *in vivo* heat shock kinetics of induction and recovery

experiment with striated muscle; and 3) and in vivo heat shock temperature-response experiment with gill, heart, and striated muscle.

The statistical design of the first study employed a complete randomized design using seven temperature treatments (25, 28, 31, 34, 37, 40, and 43°C) with three replicates. Striated muscle (50 mg) from individual fish was excised and sliced. Slices were incubated 1 hour at appropriate temperatures in physiologic fish saline. Subsequently, tissue slices were labeled with a final concentration of 2 uCi/ul L-methionine and L-cysteine (Trans ^{35}S-label, ICN) for 2 hours. Incorporation of radioactive amino acids was halted by placing samples in a -80°C freezer.

Time to induction and recovery from an in vivo heat shock exposure was determined by placing fish into 34°C (heat shock) water for 1 hour and subsequently placing them into 25°C (recovery) water for up to 11 hours. Prior to this experiment, the maximum sublethal heat shock in vivo was determined to be 34°C. Treatments were time samples at 0, 0.5, and 1 hours during heat shock, as well as, 1, 3, 7, and 11 hours post heat shock. All treatments had three replicates of three fish. 48.8±2.8 mg of pooled striated muscle was sampled and labeled with 2.0 uCi/ul for 2 hours.

The in vivo temperature-response experiment utilized a complete randomized design using six temperature treatments (25, 28, 31, 32, 33, 34°C), five replicates, and three fish per treatment replicate. Pooled tissue slices from each treatment replicate (45.9±7.8 mg gill, 49.5±3.4 mg heart and 4.7±2.1 mg striated muscle) were labeled with 1.0 uCi/ul of Trans ^{35}S-label for 2 hours.

Tissues were prepared for electrophoresis in the following manner. Thawed samples were centrifuged at 13,000 g and 4°C for 30 min. The resulting supernatant was discarded, and the pellet was resuspended in fish saline and centrifuged at 13,000 g for 20 min. The supernatant was discarded, and the pellet was suspended in 100 ul of homogenization buffer and homogenized. Samples were again centrifuged at 13,000 g for 30 min. The protein concentration of the final supernatant was then determined by the Protein-Assay Method (BIO-RAD).

Polyacrylamide gels (12.5 or 15.0%) were loaded with 30 ug of protein per lane and electrophoresis was run according to (Laemlli, 1970). After electrophoresis, gels were impregnated with ENHANCE (New England Nuclear) and exposed to KODAK XAR-5 X-ray film for 6 days. Relative intensities of bands on the film, reflecting amounts of stress protein synthesis, were quantitated using a laser densitometer.

Kruskal-Wallis nonparametric analysis of variance was used to determine the significance of the treatment effects. Comparisons

to controls, or time zero for the kinetics experiment, were determined by one tailed Dunnett T tests on ranked data.

Sodium Arsenite Exposures

Determination of the stress protein response to sodium arsenite followed a two phase procedure. The first phase was to determine the time to maximal induction of the stress protein response and the second phase employed a 96 h toxicity test in which concentration responses (including sp and toxic endpoints) were evaluated.

Sodium arsenite was found to be acutely toxic to 90 day old fathead minnows at 25 mg As/L (nominal concentration) via preliminary range find tests (first signs of mortality began at 10h). This concentration was used to determine the time of induction.

Seven 5 (19.3 L) gallon aquaria filled with 10L of dechlorinated tap water each at 25 mg As/L (nominal concentration) were prepared. Three fish were placed into each aquarium at the same time, and were sampled at intervals of : 0, 2, 4, 6, 8, 10, and 12h. Striated muscle and gill tissue were excised from all three fish from a specific tank and subsequently pooled. Stress protein induction was determined using methods described above for heat shock with the following exceptions: 1) tissue pools were incubated at 25°C for 2h with ^{25}S-methionine and cysteine (Trans ^{25}S-label, ICN) at 2.0 uCi/ul, and gels were exposed to KODAK XAR-5 X-ray film for 48h.

The toxicity test with sodium arsenite utilized a randomized design using five concentrations (6.0±0.2, 9.6±0.1, 17.7±1.9, 26.7±1.8, 35.6±2.6) mg As/L plus control, five replicates, and 13 to 15 fish per treatment replicate. Three fish were sampled per tank at 8h after initiation of the test for stress protein determination (8h was found to be the time needed for maximum level of induction). Pooled tissues from each treatment replicate (45.8±6.6 mg gill and 47.8±4.9 mg muscle) were assayed for stress protein synthesis as above. The remainder of the fish in each tank were allowed to be exposed for the entire 96 h.

Concentrations of arsenic were determined by atomic absorption spectroscopy using EPA method 206.2. Dissolved oxygen levels ranged from 4.6 to 8.8mg O_2/L, and pH ranged from 7.2 to 7.5. LC50 was determined by probit analysis. Intensities of stress protein sysntheses were analyzed nonparametrically due to heteroscadicity. Kruskal-Wallis analysis of variance was used to determine the significance of treatment effects (a = 0.05) on specific stress protein intensities. Comparisons to controls were determined by one-tailed Dunnett T tests on ranked data. Spearman rank correlations were used to compare percent mortality to stress protein intensities.

Results

Heat Shock Exposures

Synthesis of four bands with molecular weights of 100, 90, 78, and 70 kilodaltons were found in the *in vitro* heat shock study with striated muscle. Temperature significantly affected the stress protein response of sp's 100, 90, and 70 (Kruskal-Wallis, $p<0.05$). Temperature did not affect the rate of synthesis for sp 78. Dunnett's T test on ranked intensities showed significant increases ($p \leq 0.05$) in the syntheses of sp's 70 and 90 above control levels at 34 and 37°C. Sp 100 synthesis was significantly greater than control levels at only 37°C. Maximal synthesis for all sp's was at 37°C (Figure 1). Only sp's 90 and 70 were synthesized at temperatures below 37°C. Synthesis of all sp's at 40 and 43°C decreased to or below control levels. The ordered ranking in amount of synthesis of the heat shock proteins was 70 > 90 > 100 > 78. In addition, constitutive synthesis of sp 70, but no other sp's, were measured at 25°C.

Induction of the stress protein response *in vivo* with striated muscle occurred within minutes of heat shock (Figure 2). At 0.5 hours, sp's 78 and 70 showed significant increases in synthesis beyond that at 0-hour (Dunnett T test, $p \leq 0.05$). By 1 hour of heat shock, three of the four sp's detected (90, 78, and 70) showed significant increases. Syntheses of these three proteins were also maximum at 1 hour. Syntheses of all four proteins were evident at 1 hour post heat shock, where sp's 90, 78, and 70 syntheses were significantly greater than at 0-hour levels. However, sp 100 synthesis was not significantly greater than at 0-hour due to heteroscedacity. By 3 hours, post heat shock levels of syntheses of sp's 100, 90, and 70 had decreased to levels not significantly greater than 0-hour levels. Beyond 3 hours, syntheses of all sp's had recovered to near or below 0-hour levels.

Results of the *in vivo* temperature-response experiment for muscle, gill, and heart tissue are summarized in Figures 3-5. Temperature significantly affected sp synthesis for all four sp's found in the muscle (Kruskal-Wallis, $p \leq 0.05$). Increased syntheses of the sp's were not evident until 31°C (Figure 3). Significant increases above control levels were seen for sp's 78 and 90 from 31-34°C (Dunnett T test, $p \leq 0.05$). Significant increases in synthesis of sp 70 occurred from 32-34°C. Sp 100 showed significantly increased synthesis only at 32°C, because synthesis was inadequate for quantification in three of the five replicates at 33 and 34°C. Ranked order of sp responses versus temperature echoed the pattern seen in the two previous studies, sp 70 > 90 > 78 > 100. In addition, only sp 70 was constitutively synthesized at the control temperature (25°C).

The sp response was different with gill (Figure 4). Six sp's were quantified, the four found in muscle and two new ones, sp's 60 and 68. Temperature significantly affected the sp response in five of the six sp's, with an ordered response of sp 100 > 90 > 78 > 70 > 68 (Kruskal-Wallis, $p < 0.05$). Significant increases in synthesis from 25°C levels were 28-34°C for sp 68, 31-34°C for sp 70, 28-34°C for sp 78, 32-34°C for sp 90, and 34°C for sp 100 (Dunnett T test, $p < 0.05$). Maximal synthesis for sp's 68, 70, 78, and 100 was at 34°C. Sp's 68 and 70 dominated the sp response, followed by 90 > 78 > 100 > 60. As with muscle, sp 70 was constitutively synthesized at 25°C, while its doublet sp 68 was not. The intensity of the sp response in gill was about three times greater than in muscle and can be observed in the intensity scale differences between Figures 4 and 5.

Insufficient fathead minnow tissue and resultant protein was obtained to quantitatively and statistically evaluate the sp response in the heart. Thus, all fish hearts from respective temperature treatments were pooled. The qualitative response pattern is shown in Figure 5. A new heat shock protein, sp 96, was found. The sp response pattern was similar to that found in the striated muscle. Sp 70 dominated the response followed by sps 90, 76, 96, and 100. Maximum synthesis of all sp's was at 34°C. As with the other two tissues, only sp 70 was constitutively synthesized at the control temperatures.

Sodium Arsenite Exposures

The 96h LC50 for sodium arsenite was 10.0 mg As/L. Maximal induction of the stress protein response to sodium arsenite occurred by 6h and remained high through 12h of exposure.

Six stress protein bands with molecular weights of 74, 72, 70, 40, 30, and 20 kd were found in the gill (Figure 6). Arsenite treatments significantly affected the stress protein response of sp's 74, 72, 70, 40, and 20 (Kruskal-Wallis, $p \leq 0.05$). Arsenite did not affect the synthesis of sp 30 ($p=0.16$). Dunnett's T tests on ranked intensities showed significant increases ($p \leq 0.05$) in the synthesis of sp's 70, 40, and 20 above control levels from 9.6 through 35.6 mg As/L. Increased synthesis of sp20 was evident at 6.0 mg As/L yet was not significant due to high variance. Significant increases in syntheses of sp's 74 and 72 occurred from 17.7 to 35.6 mg As/L. The appearance of sp's 74 and 72 occurred at 9.6 mg As/L, unfortunately their significance was compromised due to high variance. High levels of synthesis of sp 30 were evident from 17.7-35.6 mg As/L, however only 17.7 mg/L levels were significantly greater than control levels. The ordered ranking of the spr was 20 > 70 > 72 > 74 > 40 > 30.

A similar yet less intense pattern of sp synthesis was noted in the muscle (Figure 7). Two different sp's were found to be induced in the muscle versus the gill: sp's 90 and 68.

Significant increases in sp syntheses above control levels were found in all sp's at 26.7 mg As/L. Only sp's 70 and 30 showed significant increases below 26 mg As/L. However, sp 20 appeared as low as 9.6 mg As/L. The ordered ranking of responses versus concentration was sp 70 > 20 > 30 > 90 > 68.

Stress protein intensities were correlated to 96h percent mortalities within each treatment replicate. In the gill, syntheses of sp's 74, 72, 70, and 20 were significantly correlated with mortality. However, in the muscle only sp 70 was significantly correlated with mortality. The lack of sp response from sp's 90, 68, 30, and 20 at 35.6 mg As/L greatly affected the correlation.

Preliminary Results from the First Phase of the Field Validation Site Selection and Toxicity Determination

Site Selection

Potential field validation sites ere evaluated in June 1989. Soldier Creek, Midwest City, Oklahoma was selected as our aquatic system. This creek emanates from Tinker Air Force Base and consists of industrial/sewage effluent. Soldier Creek is the largest tributary of Crutcho Creek, which empties into the North Canadian River. Six Soldier Creek sites, five Crutcho Creek, and one North Canadian River site were evaluated. Each of the Soldier Creek sites were approximately 1 mile apart with the first one less the 1/16 mile downstream of Tinker Air Force Base. One of the Crutcho Creek site was immediately below the Soldier Creek confluence. The one North Canadian Site was 1/2 mile upstream from the Crutcho Creek confluence.

Water was collected from each site and transported on ice to UNT. Toxicity of the water samples was determined by placing 90d fathead minnows into 1-gallon jars filled with 3L of water. Two sites immediately below Tinker Air Force Base (AFB) exhibited acute toxicity with total mortality occurring within 30 minutes of test initiation. However, all other Soldier Creek sites, Crutcho Creek, and North Canadian River sites showed no toxicity. The free chlorine level in the site immediately below Tinker AFB (site no. 1) was 1.6 mg Cl_2/L, site no. 2 was 0.8 mg Cl_2/L, and $\leq$ 0.2 mg Cl_2/L at all other sites. Thus, a toxicity and chemical gradient does exist in Soldier Creek.

Sediment collected at four sites in Soldier Creek, closest to Tinker AFB, smelled of fuel and solvents. Sediment toxicity was evaluated by placing 90d fathead minnows into 1 gallon glass jars filled with 600 ml of Soldier Creek sediment overlaid with 2400 ml of Soldier Creek water. No toxicity was seen in any of the stations.

Stress Protein Assay via Soldier Creek Water and Sediment/Water Exposures

Pilot time to induction studies with 90-120d fathead minnows exposed to both Soldier Creek water and sediment/water were performed. No induction of any stress proteins were evident from either exposure. This tends to indicate that the radioisotope method for determining stress protein synthesis is not sensitive enough to detect changes in stress protein composition via sublethal or subacute exposures. However, further tests with antibodies to the various stress proteins may provide the sensitivity needed for more chronic exposures.

Preliminary Results from Exposures to Sodium Chromate

Time to Induction Experiment

150 mg Cr/L induced a 20kd protein that reached maximum synthesis by 10 to 12 h. Sp 70 was not induced, especially in comparison to previous heat shock and sodium arsenite responses.

Currently, a concentration response experiment is being planned for sodium chromate using concentrations ranging from 10 mg Cr/L to 150 mg Cr/L.

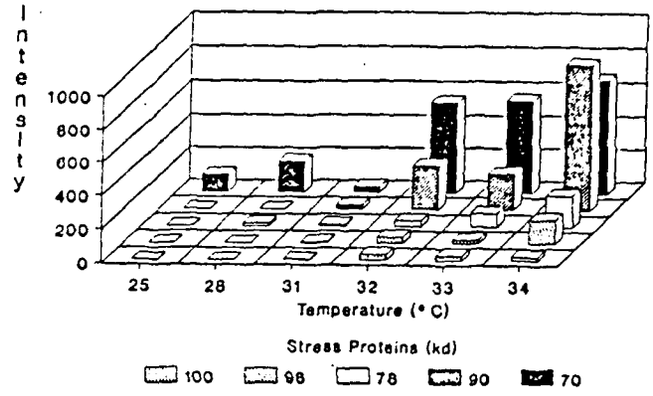

Figure 5. Intensities of the stress protein response in the hearts of fathead minnows exposed to different temperatures.

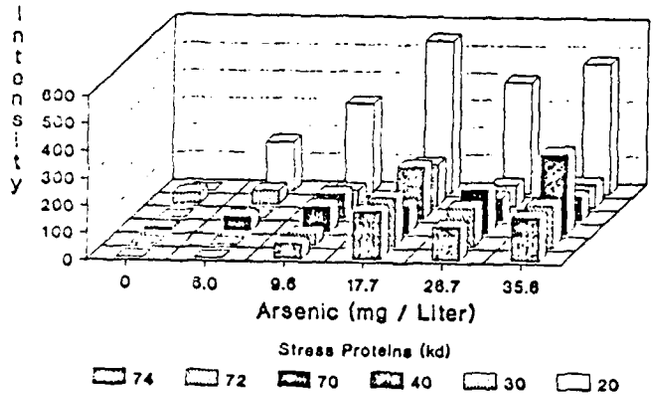

Figure 6a. Median intensites of the stress protein response in the gills of fathead minnows exposed to different concentrations of arsenic in the form of sodium arsenite.

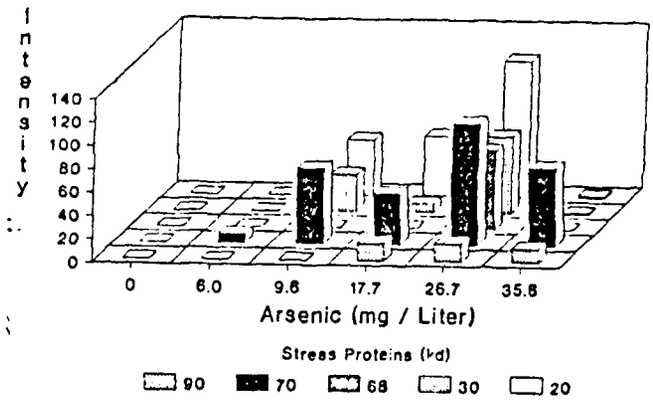

Figure 6b. Median intensities of the stress protein response in the muscle of fathead minnows exposed to different concentrations of arsenic in the form of sodium arsenite.

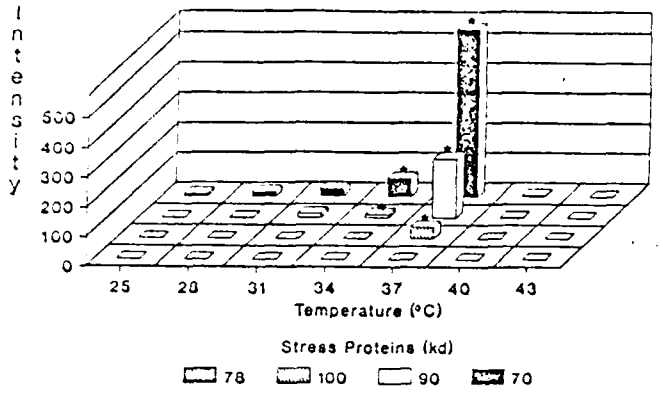

Figure 1. Median intensities of stress protein syntheses in muscle tissue, in vitro exposure, versus temperature. Asterix denote significance from 25°C (Dunnett T Test, p=0.05).

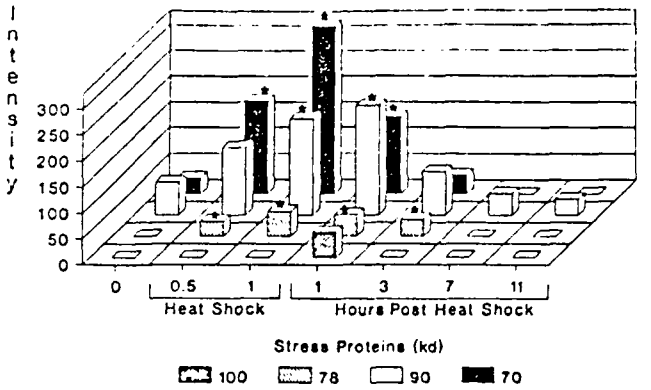

Figure 2. Median intensities of stress protein syntheses in muscle from the in vivo heat shock kinetics of induction and recovery experiment. Asterix denote significance from respective stress protein syntheses at 0h.

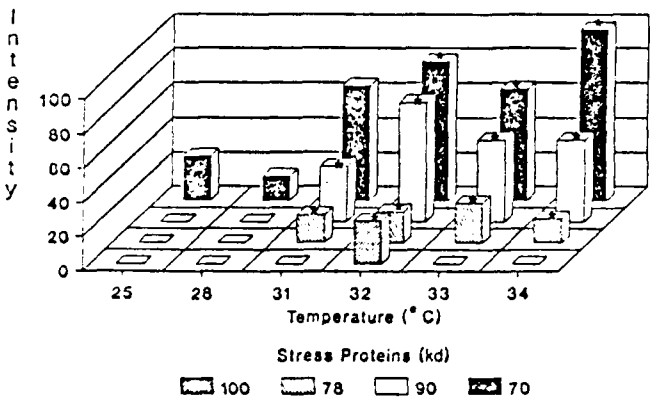

Figure 3. Median intensities of the stress protein response in muscle from fathead minnows exposed in vivo to different heat shock temperatures. Asterix denote significance from 25°C (Dunnett T Test, p=0.05).

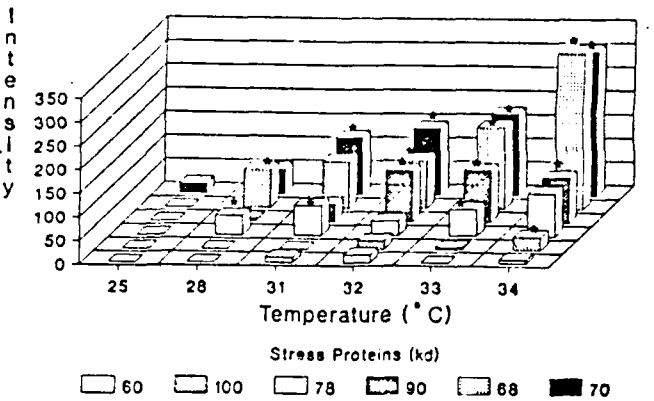

Figure 4. Median intensities of the stress protein response in the gills of fathead minnows exposed in vivo to different heat shock temperatures. Asterix denote significance from 25°C (Dunnett T Test, p=0.05).